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14. ABSTRACT

We have shown that obesity increases tumor burden without significantly altering tumor morphology suggesting that the primary effect of obesity is on tumor cell proliferation. Interestingly, the origin of the obesity did not have an effect as genetic obesity due to leptin mutation or diet-induced obesity due to a high fat western diet equivalently increased tumor growth. While there was no difference in macrophage infiltration into the tumors themselves, we found a significant increase in the proinflammatory M1 macrophage population in the obese mammary fat pad. Consistent with this increase, we observed increased expression of TNF-α in the fat pad and we subsequently showed that TNF-α enhances tumor cell proliferation in vitro. Thus our results point to the ability of obesity to create a microenvironment that is conducive to tumor growth rather than an effect of obesity on the tumor per se. Further studies are likely to reveal the mechanisms underlying the increased risk for breast cancer in postmenopausal women and identify potential targets for therapy.

15. SUBJECT TERMS
Obesity, Breast Cancer, Inflammation

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INTRODUCTION:

One in eight women will be diagnosed with breast cancer during their lifetime. There is abundant evidence that obesity confers increased risk of breast cancer. Breast cancer is strongly associated with obesity and hyperinsulinemia in post-menopausal women not on hormone replacement therapy and, furthermore, the Metabolic Syndrome is associated with a higher incidence of aggressive triple negative breast tumors. Many of these findings have been confirmed in rodentsbut the mechanisms of obesity-induced breast cancer risk remain poorly understood. We have found that the anti-inflammatory and insulin sensitizing effects of omega-3 fatty acids (ω3 FAs) are mediated by a specific G-protein coupled receptor, GPR120. Due to the potential link between obesity, inflammation, insulin resistance and breast cancer risk in post-menopausal women, we propose that this same receptor GPR120 is the critical mediator of the protective effects of ω3 FAs in breast cancer. Our central hypothesis is that ω3 FAs signal through GPR120 to reduce inflammation, attenuate insulin resistance and inhibit breast cancer growth.

We proposed two specific aims to test this hypothesis that combine studies using orthotopic tumor cell transplants and spontaneous tumors in obese wild-type and GPR120 knockout mice. **Specific Aim 1** was to test the hypothesis that ω3 FAs inhibit tumor *growth* in obese mice and that knockout of GPR120 in the host mice will abrogate these effects. This aim used a syngeneic orthotopic transplant model in which Polyomavirus middle-T antigen expressing mammary epithelial cells are injected into mammary fat pads of WT or GPR120 KO mice on normal or ω3 FA-supplemented diets. **Specific Aim 2** was to test the hypothesis that ω3 FAs inhibit tumor *initiation* in obese mice and that knockout of GPR120 will abrogate these effects. This aim will use spontaneously occurring tumors in PyMT-WT and PyMT-GPR120 KO mice to study tumor initiation. All of these studies use ovariectomized female *ob/ob* mice to create obesity-induced inflammation and insulin resistance and to simulate post-menopausal hormone levels. The control group are fed standard rodent chow and the test group receive standard diet supplemented with ω3 FA (EPA and DHA) while preserving total calories from fat.

BODY:

Task 1: Treating mice with omega3 FAs in orthotopic injection model (Months 1-18)

To test the hypothesis that ω3 FAs inhibit tumor growth in obese mice and that knockout of GPR120 in the host mice will abrogate these effects. Our initial studies have focused on validating our mammary tumor model in the obese mouse and comparing the proposed *ob/ob* genetic model of obesity to a diet-induced model. One of the important questions that we wished to address is whether the composition of the diet is important. The diet-induced model uses high fat in the diet to increase caloric intake and adipose deposition. The lipid composition of the adipose tissue is to a certain extent affected by the ingested food as lipids can be incorporated directly into triglyceride deposits. The *ob/ob* mouse becomes obese on a standard low-fat high-complex-carbohydrate rodent chow. This model relies on de novo lipid synthesis in the liver and fat to store excess nutrients as adipose tissue.

Since postmenopausal obesity is a risk factor for breast cancer, we ovariectomized (OVX) female *ob/ob* mice, heterozygous *ob/+* (Het) mice, or wild-type (WT) mice then injected Py230 tumor cells into the mammary glands (n = 7-11 mice per group). We included an 8-week high fat diet (HFD) feeding to see if genetic and diet-induced obesity were additive. Importantly, *ob/ob* mice fed a normal chow diet (NC) had significantly greater tumor burden compared to Het and WT mice (Fig. 1). The effect of genetic obesity was equivalent to diet-induced obesity as Het and WT mice on HFD had a similarly increased tumor burden. While the *ob/ob* mice tended to be even more obese when given a HFD (Fig. 2), the tumor burden was not significantly greater than the *ob/ob* mice on normal rodent diet indicating that the tumor-promoting effects are not additive. Regression analysis of the 220 tumor weights collected versus the mouse body weight (Fig. 3) revealed that the mammary tumor burden was positively correlated with the body weights of the mice ($p < 0.001$). These results indicate that the female OVX *ob/ob* mouse is a good model for postmenopausal obesity-linked breast cancer, providing validation for studies of the effects of obesity on human postmenopausal breast

cancer. Furthermore, they support the important conclusion that it is obesity, *per se,* and not simply the HFD that confers the increased tumor risk.

It was noted in the 2012 annual report that the ω3 FA diet introduced a confounding variable into the experiment as the mice did not eat equivalent amounts as the control animals. The diet had a different texture and we encountered problems with taste aversion and oxidation of the ω3 Fas. We investigated alternative formulations with different suppliers to identify a more palatable ω3 FA diet for the mice in our study. A key finding was that switching a lard-based diet to a cocoa butter based diet eliminated the feeding differences. The cocoa butter diet gives a more solid food pellet than the lard-based diet and is more palatable to the mice. Mice on a diet rich in ω3 FAs have a reduced tumor burden (Fig. 4). Importantly, whereas mice on the previous fish oil diet (FOD) were losing weight, on the current formulation mice gain and maintain weight comparable to mice on the standard HFD.

Task 2: Histological examination of mammary tumor phenotypes in the orthotopic model (months 12-18)

Our collaborator Dr. Robert Cardiff, a recognized expert in mouse mammary pathology, examined the mammary tumors and found no significant difference in the morphology of the tumors from lean or obese mice, suggesting that the increase in tumor progression was not due to a change in the tumor phenotype in the obese fat pad. It is now accepted that chronic low-grade adipose tissue inflammation is a major cause of the insulin resistance associated with obesity. This inflammation is due to an increased accumulation of proinflammatory, M1-like macrophages in adipose tissue, which release large amounts of locally-acting cytokines and chemokines and is the mechanism for obesity-induced insulin resistance. Since breast cancer arises in an adipose tissue environment, activation of similar inflammatory pathways is a plausible mechanism underlying the association between obesity and increased breast cancer risk. Recent analyses indicate that obesity is associated with local inflammation and macrophage infiltration in normal human breast adipose tissue, suggesting that this could create a microenvironment that might facilitate the initiation and/or growth of breast cancer. Staining mammary fat pads from *ob/ob* mice and WT and Het mice on HFD with F4/80 antibody indicated increased macrophage infiltration (Fig. 5). To examine inflammation in the obese mammary fat pad, we isolated stromal vascular cells (SVCs) from the mammary fat pads of lean and obese mice at the termination of the tumor growth experiment. Cells were stained with macrophage markers and analyzed by flow cytometry. The results revealed a significantly higher number of proinflammatory M1 macrophages in obese mammary fat pads (Fig. 6) and a positive correlation between M1-like macrophage infiltration and body weight (Fig. 7). Levels of specific cytokines associated with the proinflammatory activity of M1 macrophages were examined by semi-quantitative PCR. Obese fat pads showed a significantly higher expression of TNF-α than lean fat pads (Fig. 8). Levels of IL-1ß and IL-6 were very low and were not significantly different between lean and obese mice. Analysis of tumors and fat pads by immunostaining and qPCR suggests that although inflammation is increased in the mammary fat pad of HFD mice, the HFD does not affect inflammation in the tumor itself. Quantitation of macrophages in the tumors shows a slight decrease in macrophage number per tumor area and qPCR reveals no differences in the expression of proinflammatory cytokines.

Task 3: Assessment of insulin sensitivity and inflammation (months 15-18)

We have previously used an ω3 FA diet in HFD studies of inflammation and insulin resistance in male mice. We have administered the same diet to obese, wild-type, OVX female mice, but have found that the female mice do not eat adequate amounts of the ω3 FA diet and lose weight. It was noted that the diet had a different texture to the previous batch of diet, though the components were the same. Due to potential problems with taste aversion or oxidation of the ω3 FAs, we are currently investigating alternative formulations with a different supplier to identify a more palatable ω3 FA diet for the mice in our study. Luckily, obesity in the *ob/ob* mouse is driven by a lack of a leptin satiety signal in the hypothalamus. Thus, the mice are driven to eat

no matter the diet. Therefore, we tested the fish oil diet on *ob/ob* mice. Unlike the wild-type mice on the ω3-FA supplemented HFD, the *ob/ob* mice had no problem with food intake, did not lose weight, and became insulin sensitive. Thus, we have now validated the efficacy of a fish-oil diet in *ob/ob* mice. Mice on a diet rich in ω3 FAs show improved insulin sensitivity by glucose tolerance test (Fig. 9). The diet also improved cytokine profiles in visceral adipose tissue as expected from our previous studies (Fig. 10)

Task 4: Assessment of metastasis in orthotopic model (months 12-24)

We have observed that high-fat diet causes an increase in lung metastases in wild-type animals. Interestingly, this may be exacerbated by the fish-oil diet, which would be counter-indication for breast cancer prevention. This preliminary observation warrants further research as ω3 FAs are being touted for cancer prevention in the general media.

Task 5: Testing omega 3 FAs in spontaneous tumor initiation model (months 3-18)

The spontaneous initiation model was included in the original proposal in case the major effect of obesity and ω3 FAs was on tumor initiation rather than growth. We have starting breeding the PyMT mice with the GPR120 KO mice and recently generated our first PyMT-GPR120 KO mice. The PyMT needs to be bred on the male line as female mice get breast cancer and cannot breed. The male *GPR120-/-:PyMT* mice were being bred with the *GPR120-/-:ob/+* to give the *GPR120-/-:PyMT:ob/+* male mice which can then be bred with female *GPR120-/-:ob/+* mice to give the final required alleles. This is a very complicated breeding scheme as both breeders must be *ob/+* and the male must be *PyMT*, thus, female offspring with the correct alleles are only obtained in 1 out of 32 pups. Given the difficulty of obtaining sufficient mice for the proposed studies, and that the orthotopic model shows enhancement of tumor growth in the *ob/ob* background, we focused on the orthotopic model rather than the spontaneous model.

Task 6: Histological examination of spontaneous mammary tumor phenotypes (months 15-24)

As described above the spontaneous model was not pursued due to the difficulty of generating sufficient animals with the correct phenotype.

Task 7: In vitro studies of Py230 cells with LPS and FAs (months 12-24)

We postulated that TNF-α could be an important cytokine for promoting tumor progression in the obese mammary fat pad. To test this idea, we treated Py230 cells grown in vitro in media with 2 % FCS with recombinant TNF-α (Fig. 10). The results suggest that at least part of the mechanism for enhanced tumor progression in the obese mammary fat pad may involve production of TNF-α by SVCs or adipocytes and we intend to pursue this result in future studies. As we now have good data that TNF-α is a potent mitogen for Py230 cells, we will focus on TNF-α signaling effects in these cells rather than FAs or LPS. To determine the relative importance of the JNK and NFkB pathways in mediating the TNF-a effects on cell proliferation, we carried out studies using SP600-125 and JSH-23 inhibitors, specific for each pathway respectively. Our results indicate that signaling via the JNK pathway accounts for most of the observed proliferation of Py230 cells (Fig 11). Interestingly, TNF-α treatment resulted in degradation of NFkB pathway inhibitor IkBa in Py230 cells suggesting that NFkB signaling is activated in response to TNF-α in Py230 cells but does not have a major effect on proliferation (Fig. 12). Other important biologic functions such as cell survival, migration, angiogenesis, and metastasis may still require NFkB signaling. Consistent with the increase in proliferation, JNK was phosphorylated in Py230 cells treated with TNF-α, suggesting a role for JNK signaling in obesity mediated breast cancer cell proliferation.

KEY RESEARCH ACCOMPLISHMENTS:

- obesity increases tumor burden without significantly altering tumor morphology
- genetic obesity due to leptin mutation, or diet-induced obesity due to a high fat western diet equivalently increased tumor growth.
- no difference in macrophage infiltration into the tumors
- significant increase in the proinflammatory M1 macrophage population in the obese mammary fat pad
- increased expression of TNF-α in the mammary fat pad
- TNF-α enhances tumor cell proliferation in vitro.

REPORTABLE OUTCOMES:

- Results presented at American Association for Cancer Research Meeting in 2011, Abstract #152319_4
- Ellies, L.G., Johnson, A. and Olefsky, J.O. Obesity, inflammation and insulin resistance.In Energy Balance and Cancer, Vol. 7 Eds. A.J. Dannenberg and N.A. Berger. In press.

CONCLUSION: We have shown that obesity increases tumor burden without significantly altering tumor morphology suggesting that the primary effect of obesity is on tumor cell proliferation. Interestingly, the origin of the obesity did not have an effect as genetic obesity due to leptin mutation or diet-induced obesity due to a high fat western diet equivalently increased tumor growth. While there was no difference in macrophage infiltration into the tumors themselves, we found a significant increase in the proinflammatory M1 macrophage population in the obese mammary fat pad. Consistent with this increase, we observed increased expression of TNF-α in the fat pad and we subsequently showed that TNF-α enhances tumor cell proliferation in vitro via activation of JNK.

Our results indicate that the important role of obesity is to act on the stroma to create a microenvironment that is conducive to tumor growth. In contrast, obesity has little effect on the tumor per se. Further studies are needed to reveal the mechanisms underlying the effects of obesity on the stroma, but our data point to inflammatory cytokines as being an important component. Our results have suggested a possible mechanism for the increased risk for breast cancer in obese postmenopausal women and can be used to identify potential targets for therapy.

REFERENCES: N/A

APPENDICES: Supporting data is presented below.

SUPPORTING DATA:

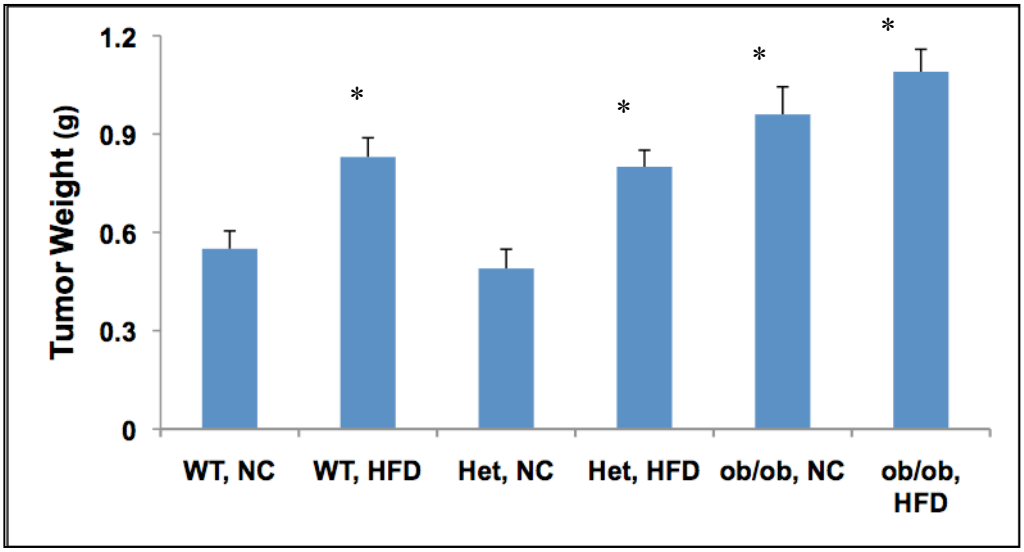


Figure 1. HFD or genetic obesity increases tumor burden in mice . ob/ob, ob/+ (Het) and wild-type (WT) mice were maintained on normal chow or placed on HFD to induce diet-induced obesity. Py230 cells were injected at week 8 after starting diet and tumors were harvested at week 15. * indicates significance vs WT or Het-NC.

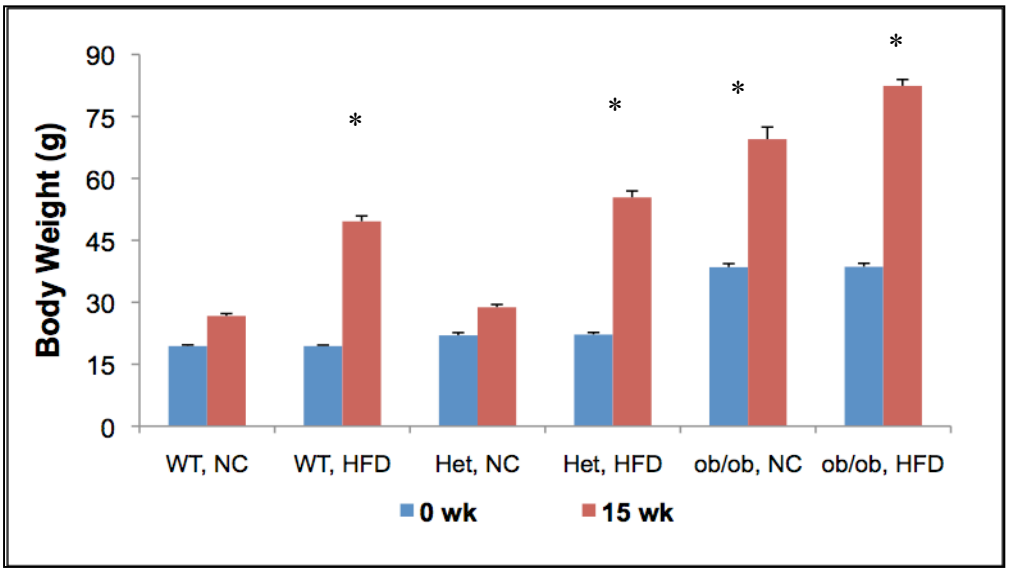


Figure 2. Body weights of WT, Het and ob/ob mice before and after 15 weeks on normal chow or HFD. * indicates significance vs time 0.

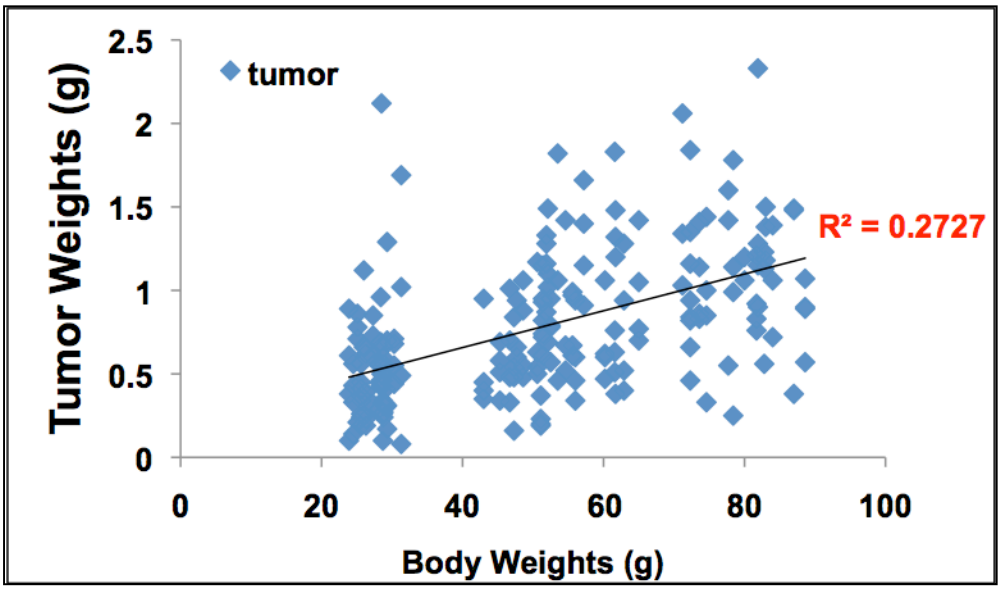


Figure 3. Correlation of tumor size and body weight for all mice in study. R = 0.522, p < 0.001

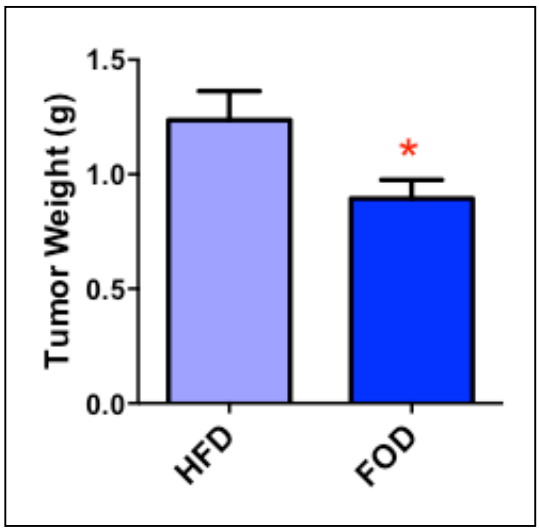
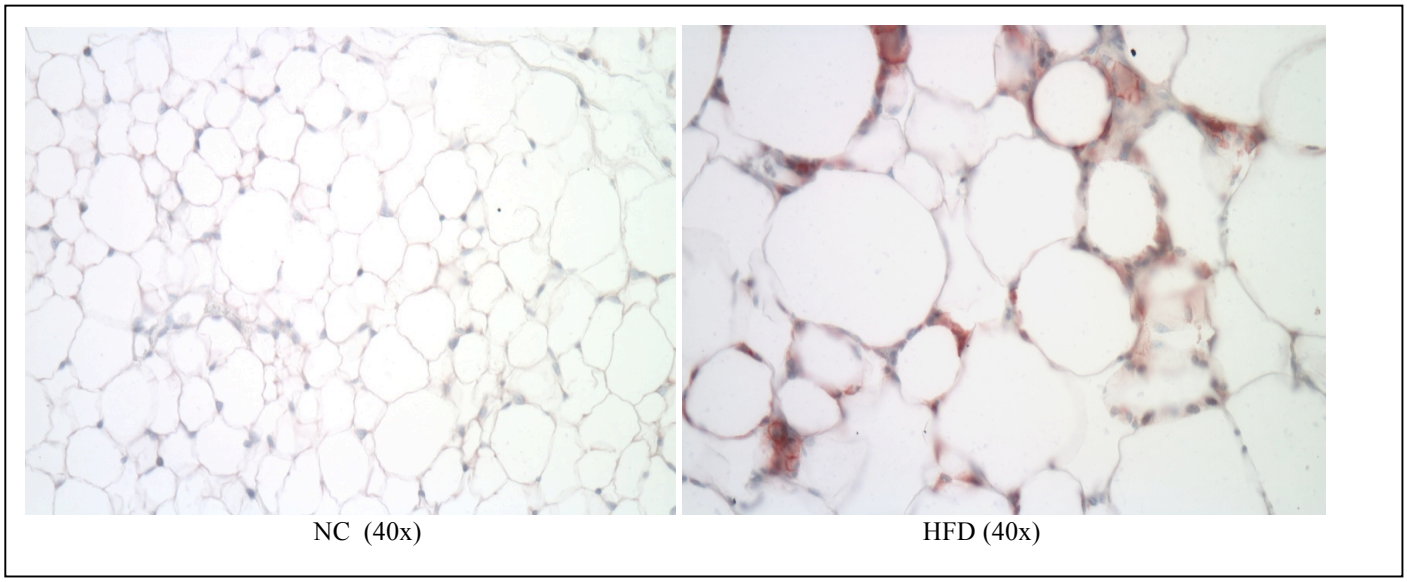


Figure 4. Effect of fish-oil diet to reduce tumor burden. Mammary tumor growth was significantly reduced in mice on the FOD compared with mice on the HFD. N=4 mice per group, $p<0.05$

Figure 5. Inflammatory macrophage infiltration into mammary fats pads is increased by obesity. Macrophages are stained using Ab F4/80 (below).



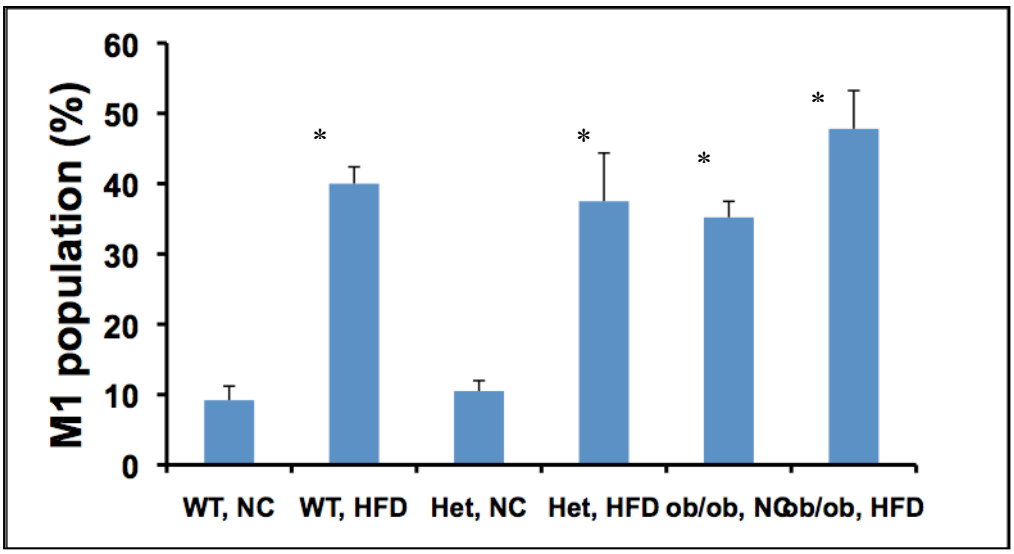


Figure 6. Inflammatory M1 macrophage infiltration into mammary fats pads is increased in ob/ob mice and in WT and Het mice on HFD by FACS, * indicate significance vs WT-NC.

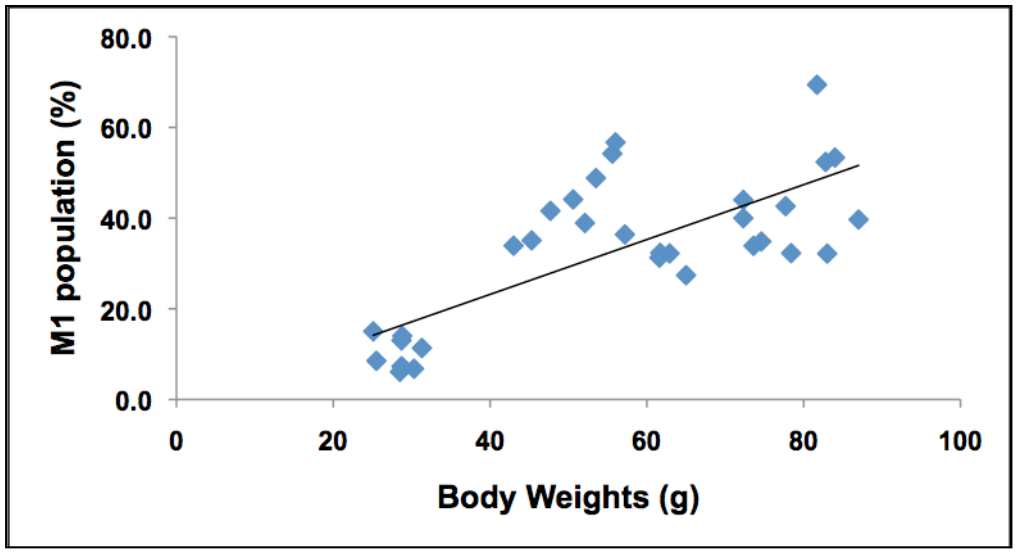


Figure 7. Correlation of M1 macrophage infiltration and body weight. $r = 0.746$, $p < 0.001$

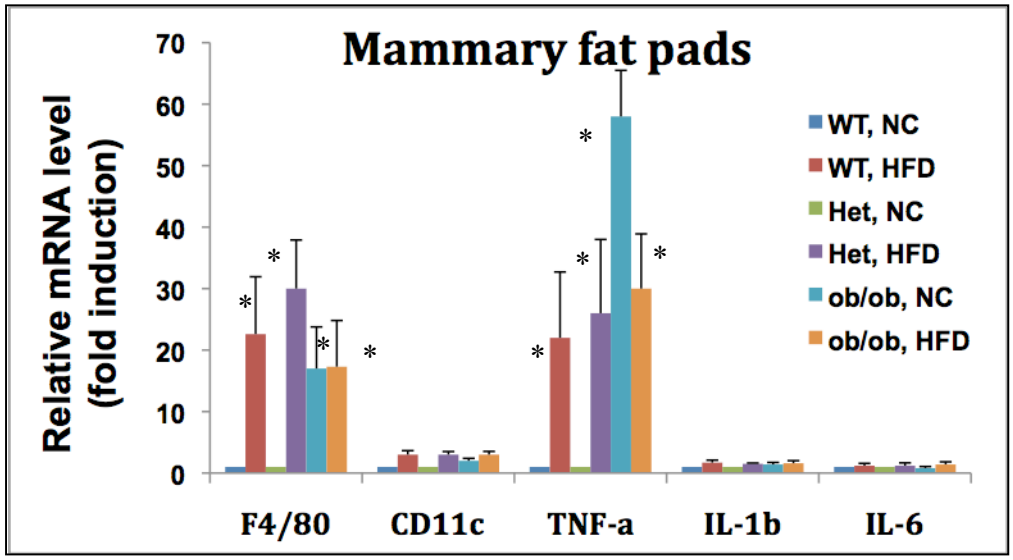


Figure 8: Inflammatory cytokine measurements in mammary fat pads from ob/ob, het and WT mice on normal chow or HFD. Cytokines and macrophage markers were measured by QPCR.

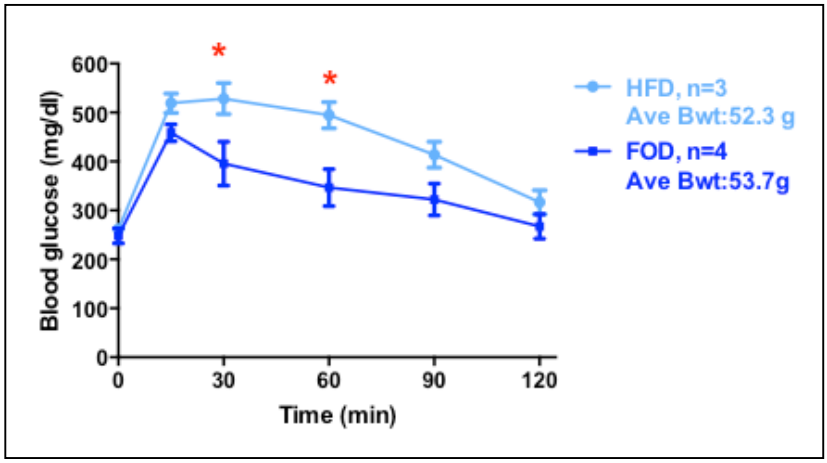


Figure 9: Mice treated with a fish oil diet (FOD) showed improved insulin sensitivity compared with mice on a standard high fat diet (HFD) in the glucose tolerance test.

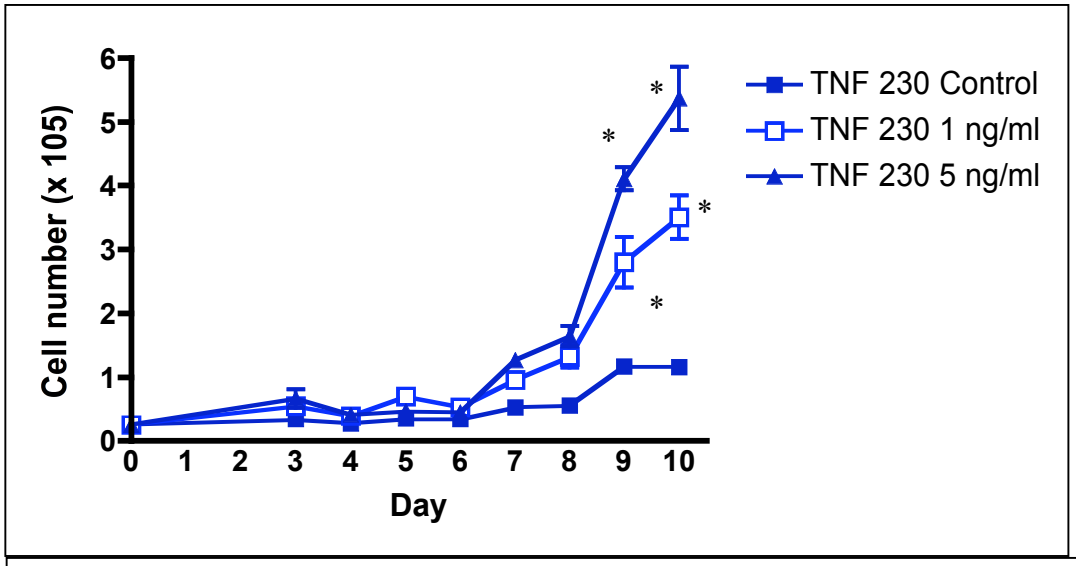


Figure 11. TNF-α is a potent mitogen for Py230 breast cancer cells..5 x 10^3 cells per well were plated into 3 x 24 well plates and treated with: Vehicle, 1 ng/ml or 5 ng/ml TNFa in triplicate. Cells were counted at the indicated times. (mean ± SEM). * indicates significance vs control

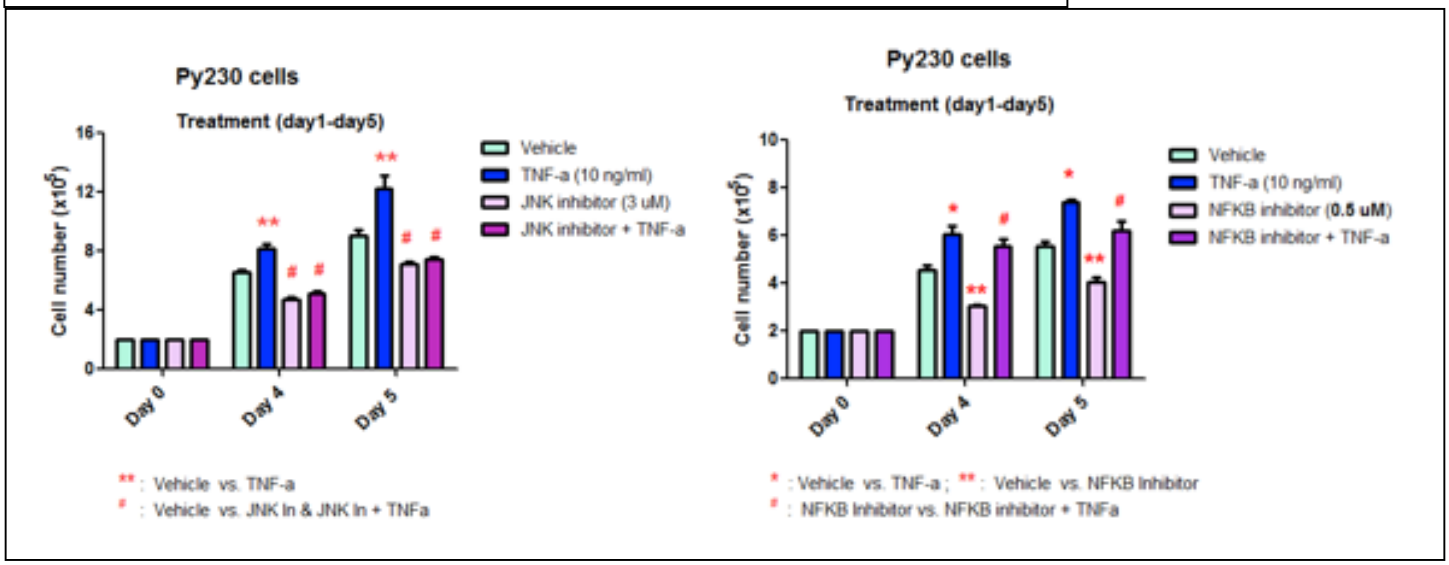


Figure 12. **Left:** Cell proliferation studies show that the proliferative effects of TNF-a on Py230 cells are mediated by the JNK inhibitor SP600-125. **Right:** The NFkappaB inhibitor JSH-23 does not affect TNF-a induced proliferation. Data are means ± SEM for triplicate samples at the indicated time points. The NFkappaB inhibitor alone significantly reduces Py230 proliferation ($p<0.01$), but does not affect the relative increase in TNF-a-induced proliferation on day 4 or 5. The JNK inhibitor significantly reduces TNF-a-induced proliferation on day 4 and day 5 $p<0.001$.

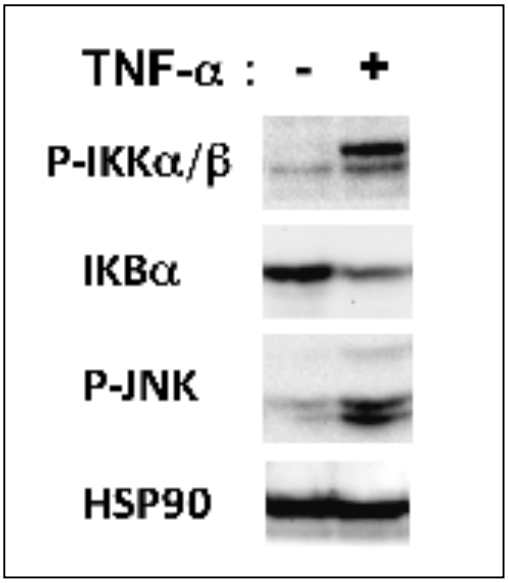


Figure 13. Py230 cells were stimulated with or without TNF-a (10ng/ml) for 15 min and then subjected to western blotting. Phosphorylation of IKKa/ß, IKBa degradation, and phosphorylation of JNK were detected in TNF-a stimulated Py230 cells.